EXPLORATIONS IN BIOMOLECULAR INTERACTIONS: EXPERIMENT AND THEORY

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ABSTRACT

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The chemistry of biology is mediated by interactions between proteins, ligands, and small molecules. Understanding the mechanism of these interactions is critical for applications in drug development and biotechnology. In the Webb Laboratory, we investigate electrostatic interactions at protein-protein and protein-surface interfaces with a particular interest in discovering the role of electrostatics in these interactions as well as exploiting the very same in order to develop new bio-inorganic hybrid technologies, such as bio-sensors. This work demonstrates an attempt to direct the immobilization of an electron transfer protein, Azurin, to an inorganic surface without relying on covalent bonding. Instead, a peptide engineered to complement the electrostatic profile of Azurin's surface is functionalized to a surface via a self-assembled monolayer and the Huisgen 'Click' reaction. Experimental methods such as infrared and mass spectroscopy are used to study the characteristics of the peptide-protein, peptide-surface, and protein-surface interactions. Likewise, computational methods were employed in order to design the peptide binding partner. Together, this work represents a step in an ongoing effort to marry computational and experimental tools in order to direct hypothesis driven science.

Explorations in Biomolecular Interactions: Experiment and Theory

I. Introduction.

The work of biology is carried out by proteins. These ubiquitous and highly specialized structures are responsible for catalyzing the chemical reactions which sustain and reproduce the fundamental activities of life at the molecular level. Digging deeper still, the primary locus of the chemistry of life is the protein surface. In an effort to better comprehend (and later exploit) the mechanisms of molecular biology, it is absolutely necessary to develop first an understanding of protein interactions. This work, carried out over two years in the laboratory of Professor Lauren Webb at the University of Texas at Austin's Department of Chemistry, utilizes computational and experimental tools to develop and study a tunable method for protein immobilization which exploits the electrostatic interactions of biological molecules.

MOTIVATIONS

There has been significant interest in developing methods of integrating the diverse, highly-specialized capabilities of proteins with the power of computer technology. This is due to the fact that biotechnologies, sensors, and catalysts can be cheaper, more efficient, and more effective at the micro scale than their inorganic counterparts. Not to mention the fact that biological molecules can carry out their functions under generally benign conditions. To this end, the development of new bio-inorganic technologies, especially biosensors, has generated a need for chemical protein immobilization methods which do not negatively affect the protein's native function. The three most popular techniques for protein immobilization used in this field of research are chemical adsorption, covalent attachment, and physical entrapment . All three techniques expose the protein to a harsh

chemical environment which often threatens to compromise its functional, native structure. Covalent attachment and chemical adsorption in particular require the presence (engineered or natural) of surface-exposed residues capable of chemical bonding with a material surface or a chemical tether. The structural resilience demanded by these approaches seriously limits the species and diversity of biological molecules which can be integrated with inorganic technology. Thus, it is necessary to develop new methods for protein immobilization which are not as structurally demanding and are generalizable to a more diverse array of potential proteins.

COMPUTATIONAL TOOLS

The most important question in chemistry is: Where are the electrons? Any attempt at understanding the structural biology of a protein must answer this question. Today, we utilize computational tools to determine the electronic structure of molecules from first principles with greater accuracy and more confidence than in previous eras. One particular method especially relevant to the work that will be presented later is the Adaptive Poisson-Boltzmann Solver (APBS). This tool can model protein surface electrostatics as well as electrostatic interactions at interfaces between different molecules or macromolecules by solving the Poisson-Boltzmann Equation (PBE). The PBE relies on the Poisson equation for an electrochemical potential in addition to the Boltzmann equation for calculating the local charge density at each distance from the surface or interface in question.

$$\nabla^2 \Psi(x, y, z) = \frac{\delta^2 \Psi}{\delta x^2} + \frac{\delta^2 \Psi}{\delta y^2} + \frac{\delta^2 \Psi}{\delta z^2} = \frac{-\rho_e}{\epsilon_r \epsilon_0} \; (Eq\ 1.1)$$

$$c_i = c_i^0 \, e^{\frac{-W_i}{k_B T}} \; (Eq\ 1.2)$$

$$\rho_e = F \sum z c_i = F \sum z c_i^0 \, e^{\frac{-z_i q_e \Psi}{k_B T}} \; (Eq\ 1.3)$$

The primary consequence of the electronic structure at any particular location on the protein surface will be the electrostatic potential at that location. Wherever the electrostatic potential is heterogenous -- that is, the potential has a nonzero gradient -- there will be an electric field. By solving for the gradient of that electric field, the PBE allows us to characterize the electrostatic environment at a protein surface; in other words, resolving where the electrons are by returning a picture of the positive and negative charge distributions on the surface. The solution to the PBE[1] can be visualized using three dimensional modeling software.

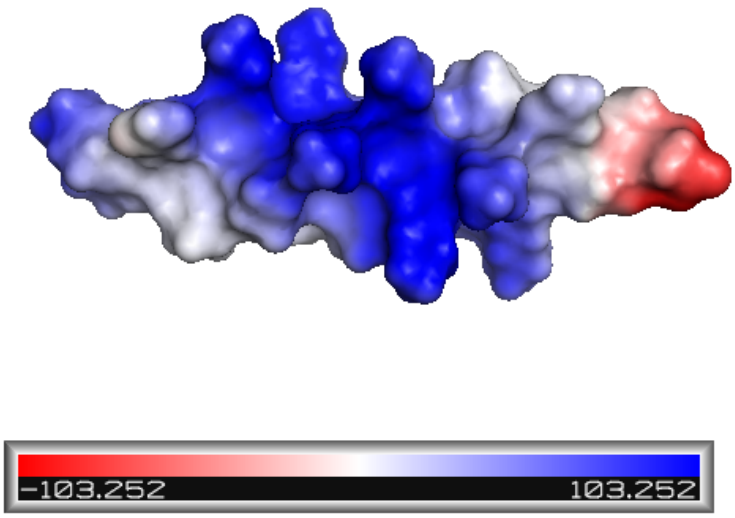


Figure 1: APBS generated electrostatic potential surface of a short, alpha helical peptide a16x. Blue regions are more positively charged while red regions are negativey charged.[2]

A MODEL SYSTEM FOR STUDYING THE INTERFACE OF BIOMOLECULAR INTERACTIONS

Millions of years of evolution have led to a tremendous diversity of protein function, much of which human ingenuity has not been able to reproduce through artificial means. In particular, biological proteins outclass synthetic materials in chemical catalysis and small molecule sensing (ligand binding). As a result, there is currently great interest in developing integrated biological-electronic materials and devices which exploit both the functionality and diversity of biological molecules and the sensing, measurement, and communication capabilities of microelectronics for medical, industrial and experimental applications. To this end, research in the Webb laboratory has attempted to develop a generalizable and highly tunable model system for studying the immobilization of proteins onto inorganic surfaces. The biomolecular structure – and therefore the function – of a surface-bound proteins is often adversely affected by the chemical environment at the surface. In solution, a solvated protein can retain its native structure and enjoy greater freedom of movement and orientation without sacrificing its function. Tethering a protein to a surface, on the other hand, might lead to aggregation at the surface, partial or total unfolding due to the physical constraints of the surface bond, or result in a static orientation that precludes the protein from binding its substrate. The approach detailed in this work aims to minimize the impact of the surface chemical environment on the protein by first covalently tethering a short peptide to the surface which has been specifically designed to complement the electrostatic profile of a protein of interest. The work herein combines computational and experimental approaches in order to develop and characterize such a system. Methods developed in the Webb laboratory have shown that while chemical binding imposes structural changes on the bound molecule it is possible for short peptides

to be successfully tethered to inorganic surfaces while still retaining their biologically functional secondary structure under certain experimental conditions. However, retaining a functional structure becomes more challenging as the size and complexity of the biological structure increase. Nevertheless, there are large proteins capable of binding substrate without significantly altering their structure -- Azurin is one such protein as research in our laboratory reveals. This research is driven by two core hypotheses: First, a surface-functionalized peptide (SFP) can be made to act as a substrate for a protein and, second, binding a protein to a surface-functionalized peptide instead of directly onto the surface itself will reduce the impact the surface environment has on the macromolecule's structure and function.

II. Methods

The core objective of this work is to develop and characterize a tunable system for protein immobilization; in other words, how to best engineer such a system and, second, understand how the relevant properties of the system can be exploited. The secondary objectives of this work are: first, the identification of a promising peptide binding partner for azurin; second, the functionalization of the peptide onto the surface; and third, the successful binding of a functional protein to the peptide binding partner.

PROTEIN, PEPTIDE AND SURFACE IDENTIFICATION

Azurin is a bacterial electron transfer protein which participates in the mitochondrial electron transfer chain, the main energy generating process for the bacterium. Azurin was chosen because its biological function -- electron transfer -- does not require a significant

structural change in the protein in order to be carried out. Furthermore, azurin's electrostatic surface profile contains a positively charged patch near the electron transfer active site which could potentially be exploited in the electrostatic immobilization process.

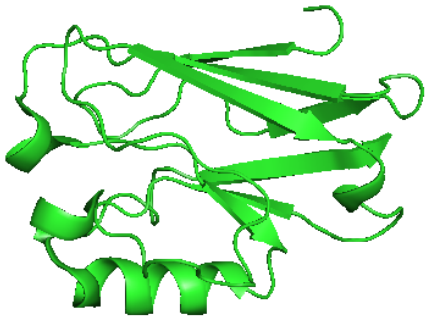


Figure 2: Monomer crystal structure of azurin. APBS of the azurin monomer reveals a hydrophobic patch at the surface of the alpha helical portion of the crystal structure. [14]

The peptide binding partner (a16x) was developed based on an alpha helical peptide previously used in the Webb Laboratory to study the retention of secondary structure following peptide functionalization with an inorganic surface. This original peptide (a20x) was also studied as a part of this project in order to replicate previous experiments. The a16x peptide consists of alanine and glutamine residues in a regular pattern only interrupted by an unnatural amino acid residue (L-propargylglycine) which is necessary for the surface functionalization reaction. A20x is nearly identical except it contains an additional four residues and the amino acid composition and patterning are different; in other words, the alpha helix is one 'turn' longer than the peptide binding partner.

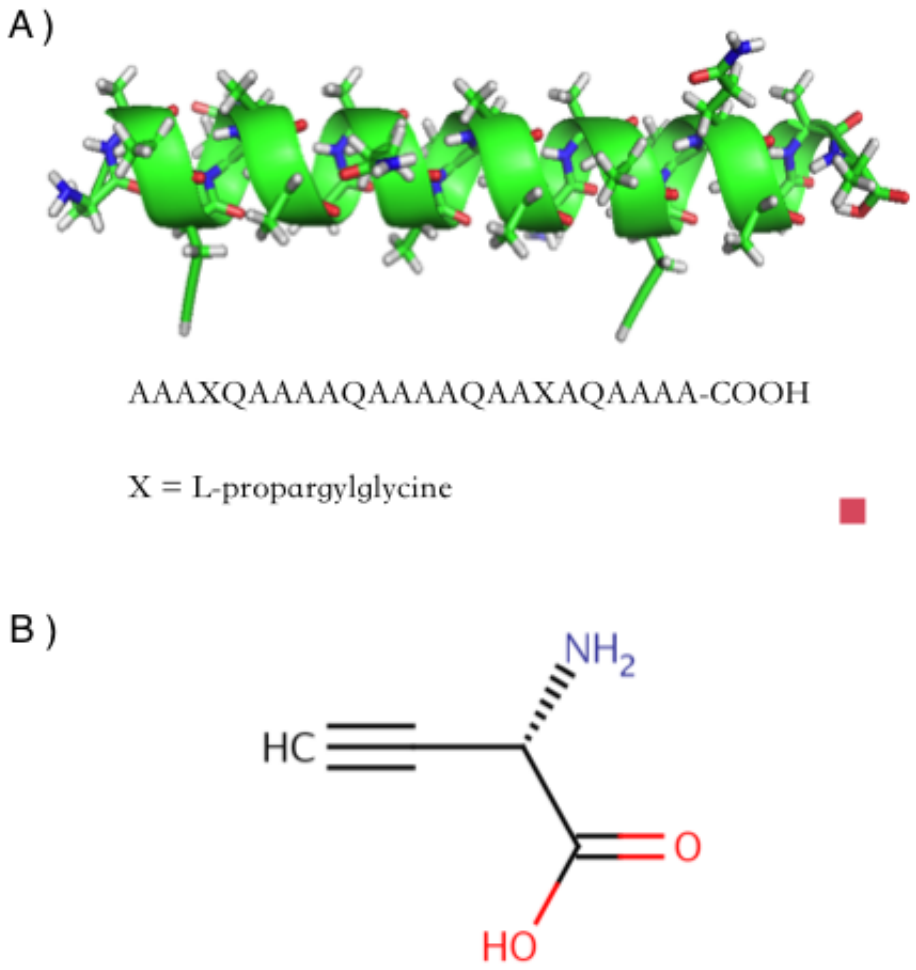


Figure 3: A) The peptide binding partner, a16x, with its amino acid sequence. B) The structure of the unnatural amino acid L-propargylglycine.

PROTEIN PURIFICATION

Azurin was purified using an e-coli vector grown in the laboratory. An Azurin plasmid containing e-coli stock was maintained in lysogeny broth. Bacterial vector containing the correct plasmid was isolated via antibiotic selection; that is, the Azurin plasmid also contains the gene for ampicillin resistance and the bacterial vector was cultured in the presence of ampicillin. After the bacterial vector was allowed to culture to the desired density, a pellet was collected following centrifugation.

The cells contained in the pellet were then lysed using hen egg lysozyme in addition to RNAse free DNAse I. The protein was collected from the supernatant of the lysed cell mixture and brought to 50 mM sodium acetate pH 4.3, and then to 20 mM copper sulfate. At this low pH Azurin is still stable but other proteins precipitate out and can therefore be separated from the solvated Azurin through centrifugation, as was done. The protein lysate was purified via fast protein liquid chromatography utilizing a cation exchange column and then stored.

STRUCTURAL CHARACTERIZATION

Circular dichroism (CD) spectroscopy was used in order to qualitatively determine the secondary structure of the peptide and protein species under investigation. In CD spectroscopy, a sample is bombarded with both clockwise (right-handed) and counterclockwise (left-handed) circularly polarized light. The chiral sample, in this case a protein or peptide, will absorb lights of different polarization directions differently. The difference between the absorption intensities of the sample corresponding to left-handed and right-handed polarized lights is measured by its CD value with units of molar ellipticity. Molar ellipticity is calculated according to the equation:

$$A_L - A_R = \Delta A$$
$$\emptyset(mdeg) = \Delta A * 32.982$$
$$\emptyset(^\circ cm^2 dmol) = 100 * \emptyset(mdeg)/(C * I)$$

Where A_D is the absorption of light circularly polarized in the D direction, C is the concentration of the sample in mols and I is the path length of the CD cell in cm.[3] The CD spectra which is then produced measures the CD value at each wavelength of light absorbed in the ultraviolet and visible light portions of the electromagnetic spectrum. These spectra can then be qualitatively evaluated based on their peak and trough structure to determine the predominant secondary structural character in the sample being investigated.

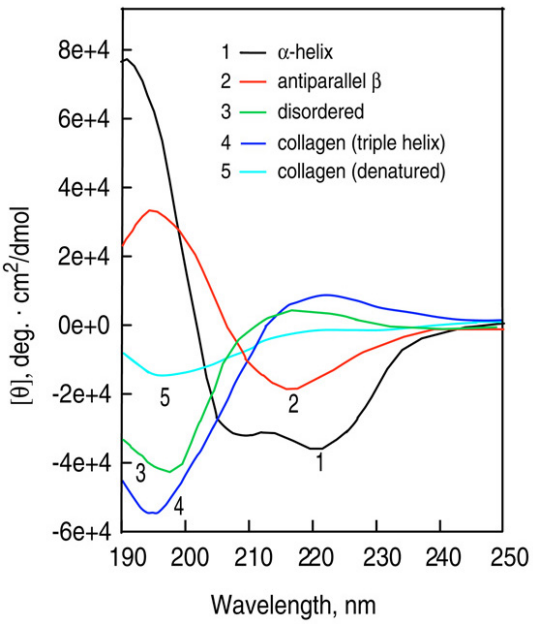


Figure 4: Generic CD spectra for characteristic secondary structures. [16]

The spectrometer utilized herein was a JASCO J-810 circular dichroism spectrometer. All of the spectra were collected with samples placed inside of a cell with a 1 mm pathlength.

In addition to CD spectroscopy, ultraviolet photon dissociation mass spectrometry (UVPDMS) was employed in collaboration with the Brodbelt Group at the University of Texas Department of Chemistry in order to determine the integrity of the three dimensional structure of the Azurin protein in different solvent environments. In UVPDMS, a laser pulse of photons with energies in the ultraviolet portion of the electromagnetic spectrum bombard a molecule of interest, in this case an aerosolized solution of azurin and peptide. The energy of the pulse is sufficient to result in fragmentation of the protein and peptide. These fragments are then analyzed via mass spectrometry. The changes in the fragmentation pattern of the protein can be used to infer changes in the three dimensional structure of the protein.

SURFACE PREPARATION

Surfaces were prepared according to the methods published in the Webb laboratory . In short, silicon wafers coated with 10 nm of chromium followed by 100 nm of gold via thermal deposition. The surfaces were then exposed to a solution of twenty-five percent 11-bromo-1-undecane thiol (BrUDT) and seventy-five percent decane thiol (DT) in ethanol for twenty-four hours, resulting in a surface coated by a ten carbon self-assembled monolayer where twenty-percent of the monolayer is bromine terminated.

FUNCTIONALIZATION OF PEPTIDE ONTO A GOLD SURFACE

The peptide is functionalized to the surface via Huisgen cycloaddition, or "Click" reaction, onto a bromine terminated self-assembled monolayer.

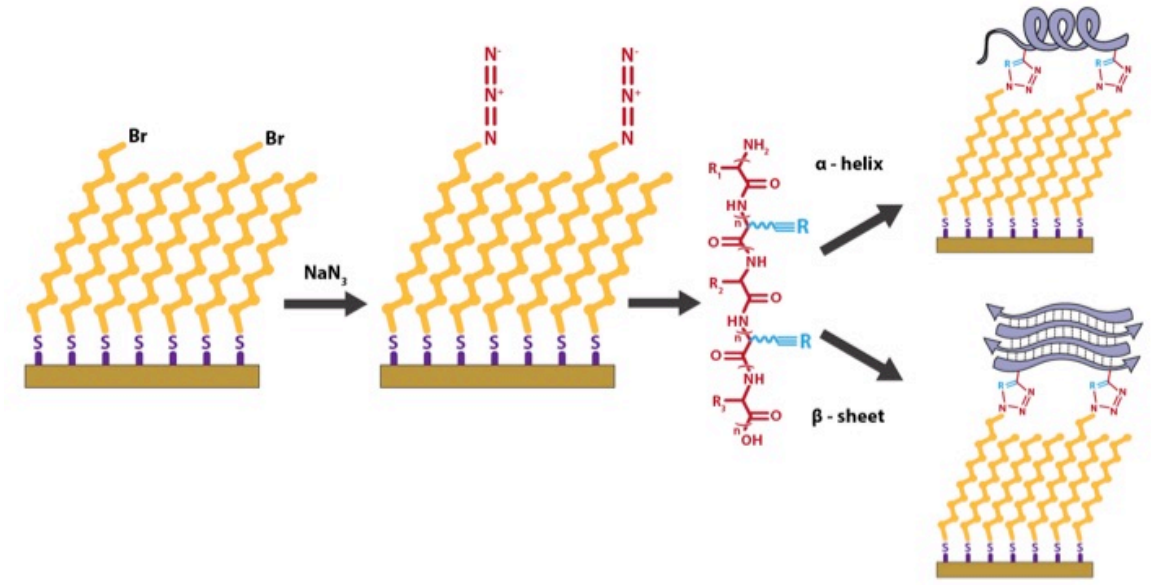


Figure 5: Huisgen cycloaddition or "Click" reaction. The self-assembled monolayer is adsorbed onto the surface and a controlled fraction of this monolayer also contains a terminal bromine. Sodium azide nucleophillically adds to the self-assembled monolayer and is then capable of binding of binding to the triple-bonded carbon functional group on the L-propargylglycine.[4]

The successful functionalization of peptide onto the surface can be confirmed via grazing incidence angle reflection−absorption infrared spectroscopy (GRAS-IR). GRAS-IR was also used to study the extent of the Huisgen cycloaddition by detecting the presence of azide on the surface samples given that azide is spectroscopically active in the infrared spectrum. Thus, the absence of the azide peak in the infrared spectra quantizes the extent of the reaction. Surface vibrational spectroscopy was collected with a Bruker Vertex 70 Fourier transform infrared (FTIR) spectrometer equipped with a A518/Q horizontal reflection apparatus for illuminating the sample at a grazing angle of 80° with respect to the surface normal. The sample chamber was continuously purged with N_2 (g), and once inside the chamber samples were transferred with an externally controlled sample manipulation arm to avoid breaking the N_2 (g) purge. The chamber was continuously purged for one hour before any measurements to reduce background noise from H2O and CO2. All measurements were made with p-polarized light. Two sets of scans were collected for each sample. For the first, a mercury cadmium telluride (MCT) detector was used to collect 100 scans between 400 and 4000 cm^-1 at a resolution of 4 cm^-1, ideal for the amide carbonyl peaks between 1500 and 1700 cm^-1. For the second, an indium antimonide (InSb) detector was used to collect 100 scans between 1870 and 4000 cm^-1 at a resolution of 4 cm^-1 to take advantage of its superior sensitivity for the N_3 absorption near 2100 cm^-1. All samples were referenced to a common clean bare gold substrate to compare absolute differences of all observed signals between different samples. After background subtraction, the baseline of each spectrum was flattened with a linear polynomial function, and the integral under the peak of interest was then computed using the FTIR spectrometer's OPUS software.

PROTEIN BINDING TO A PEPTIDE FUNCTIONALIZED SURFACE

In collaboration with the Brodbelt group in the University of Texas Department of Chemistry, ultraviolet photon dissociation mass spectrometry (UVPDMS) was used to study the binding and complexing properties of azurin in solution with either a16x or a20x. The fragmentation pattern of the Azurin protein in addition to the pattern produced by the Azurin-peptide complex can be used to determine the peptide binding locations and the level of specificity with which it binds the Azurin protein.

III. Data and Results

CONFIRMING THE INCORPORATION OF COPPER INTO AZURIN

Following purification, an absorption spectrum of the solvated Azurin protein was taken in order to confirm the incorporation of copper into Azurin utilizing a UV-Vis spectrometer.

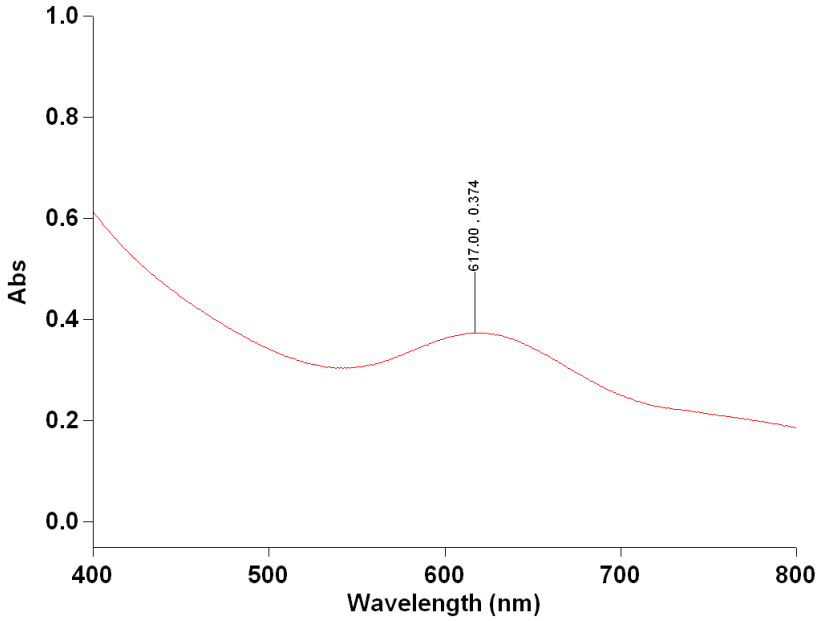


Figure 6: IR spectrum of solvated Azurin following purification by FPLC.

A clear absorption peak is visible at 617 nm which agrees with the characteristic absorption range of Cu^{2+} (600-620 nm).

STRUCTURAL CHARACTERIZATION

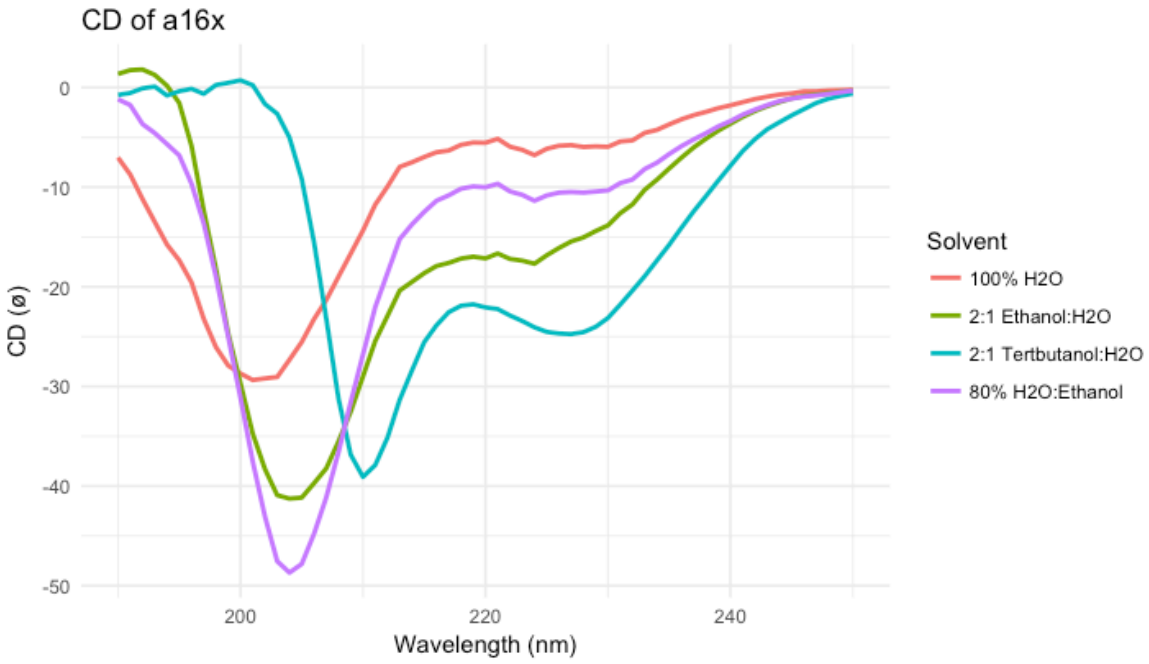


Figure 7: CD spectra of the a16x peptide in 100% H2O, 2:1 Ethanol:H2O, and 2:1 Tertbutanol:H2O solvents. The units of circular dichroism (CD) are molar ellipticity, degrees cm^2 dmol (ø).

CD spectra of the a16x peptide were collected in order to qualitatively determine its secondary structure in a variety of solvents. The concentration of the peptide in each of the solvents was kept constant throughout at 200 µM. The CD of the peptide in 2:1 tertbutanol:H2O was able to reproduce the alpha helical structure observed by Gallardo in the legacy peptide in the same solvent. Similarly, the CD of the peptide in 2:1 ethanol:H2O shows alpha helical structure. The peptide CD spectrum in 100% H2O, however, reveals a predominantly unfolded structure. Molecular dynamics simulations carried out in the laboratory by graduate student Jeremy First attempted to predict the secondary structure of the a16x peptide in water and two-to-one tertbutanol to water solvents. Those simulations reveled that a completely unfolded peptide in pure water and an alpha-helical structure which rapidly converted from folded to partially unfolded in the mixed alcohol solvent.

Given this information and the CD spectra above, it is useful to conceive the samples with CD troughs closer to 200 nm as having more unfolded character than those with two troughs near 210 and 222 nm which are characteristic of alpha-helices. In this way, molecular dynamics simulations are able to provide more insight into the secondary structure composition of the peptide in these solvents than CD alone.

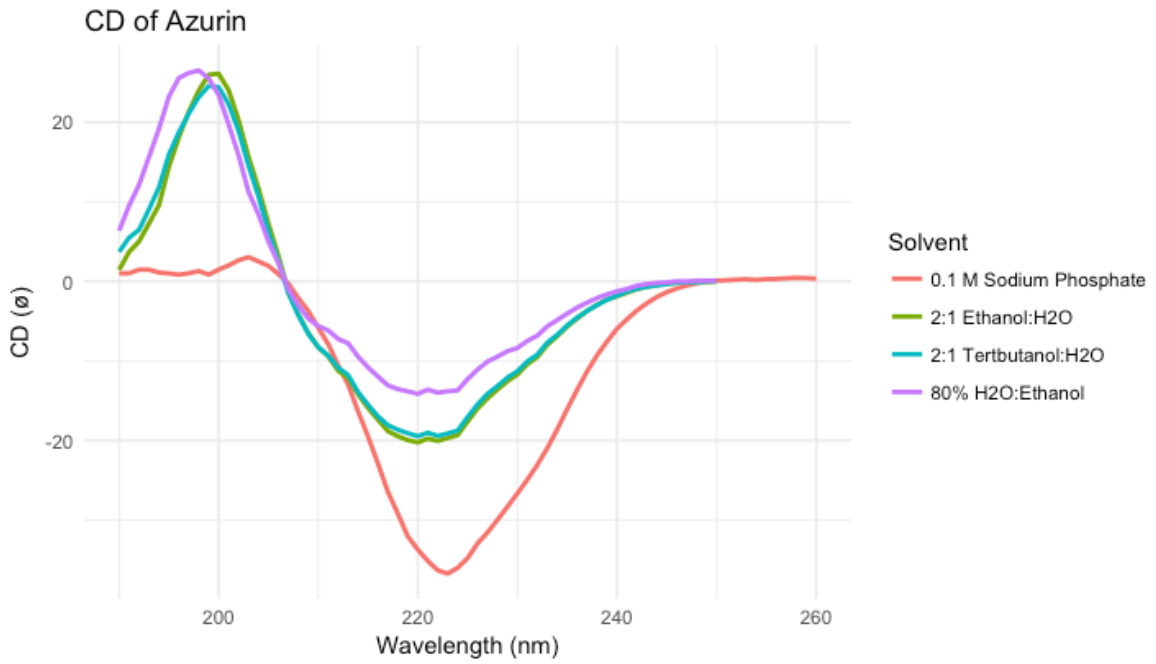


Figure 8: CD spectra of Azurin in 80% H2O:Ethanol, 2:1 Ethanol:H2O, and 2:1 Tertbutanol:H2O.

Similarly, CD spectra of the Azurin protein was collected in order to confirm the retention of secondary structure in a variety of solvents. The concentration of Azurin in each of the solvents was kept constant throughout at 5 μM, and the pH in each sample was maintained near 7.0. The solvents chosen were identical to those used in the peptide CD experiment with the aim of finding ideal solvating conditions for both the peptide and Azurin protein. An additional spectrum was also collected for Azurin solvated in 0.1M sodium phosphate in order to verify that the native secondary structure of our purified

Azurin as revealed by CD spectroscopy agreed with the literature, and it did. The peptide chains which make up the Azurin protein have a primarily beta-sheet secondary structure, as is evident from the spectrum above. The Azurin samples solvated in alcohol solutions have a considerably more mixed secondary character than the Azurin in the 0.1M sodium phosphate. The peak at 200 nm betrays some alpha helical character but the single trough near 222 nm demonstrates that primary secondary character of the protein remains beta-stranded. Further, the two-to-one alcohol to water solutions seem to have more beta-sheet secondary character of the alcohol mixtures tested.

Chris Chrittenden of the Brodbelt Group at the University of Texas at Austin collaborated with our laboratory on this project and studied the fragmentation pattern of Azurin in different concentrations of alcohol solvent via UVPD Mass Spectrometry.

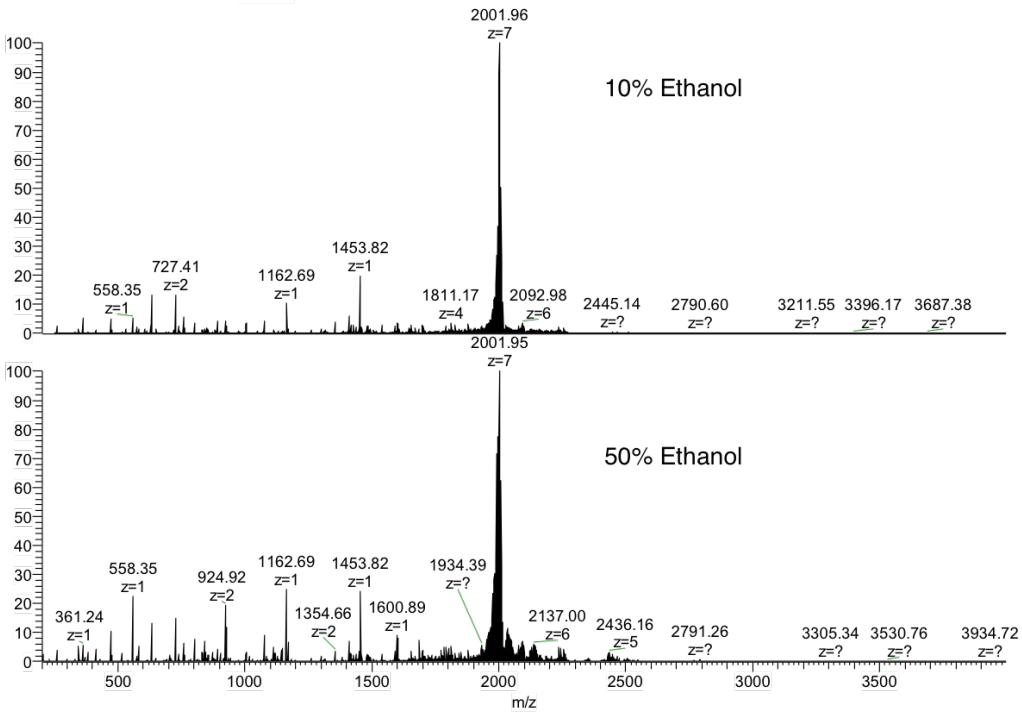


Figure 9: Fragmentation pattern of Azurin as observed via UVPD Mass Spectrometry.

The fragmentation patterns of the least and most extreme alcohol concentrations tested (10% and 50%, respectively) are not significantly different from one another. This suggests that the three dimensional structure of Azurin is also relatively unperturbed by the concentration of alcohol in the solvent. Together these data suggest that two-to-one tertbutanol and two-to-one ethanol solutions are the ideal solvents for both Azurin and peptide. Next, it was necessary to determine whether or not Azurin and the a16x peptide complexed in solution, and, if they did, to what regions of the Azurin surface did the peptide bind.

FUNCTIONALIZATION OF PEPTIDE ONTO A GOLD SURFACE

The success of the Huisgen cycloaddition or "Click" reaction responsible for tethering the a16x peptide to the SAM layer on the gold surfaces was determined using infrared spectroscopy. The "Click" reaction eliminates the azide functional groups from the SAM, and, therefore, it is possible to measure the extent of completion of the reaction by observing the disappearance of the azide peak in the infrared spectrum of the peptide-functionalized surface and the appearance of a large methylene stretch corresponding to the organic structure of the peptide.

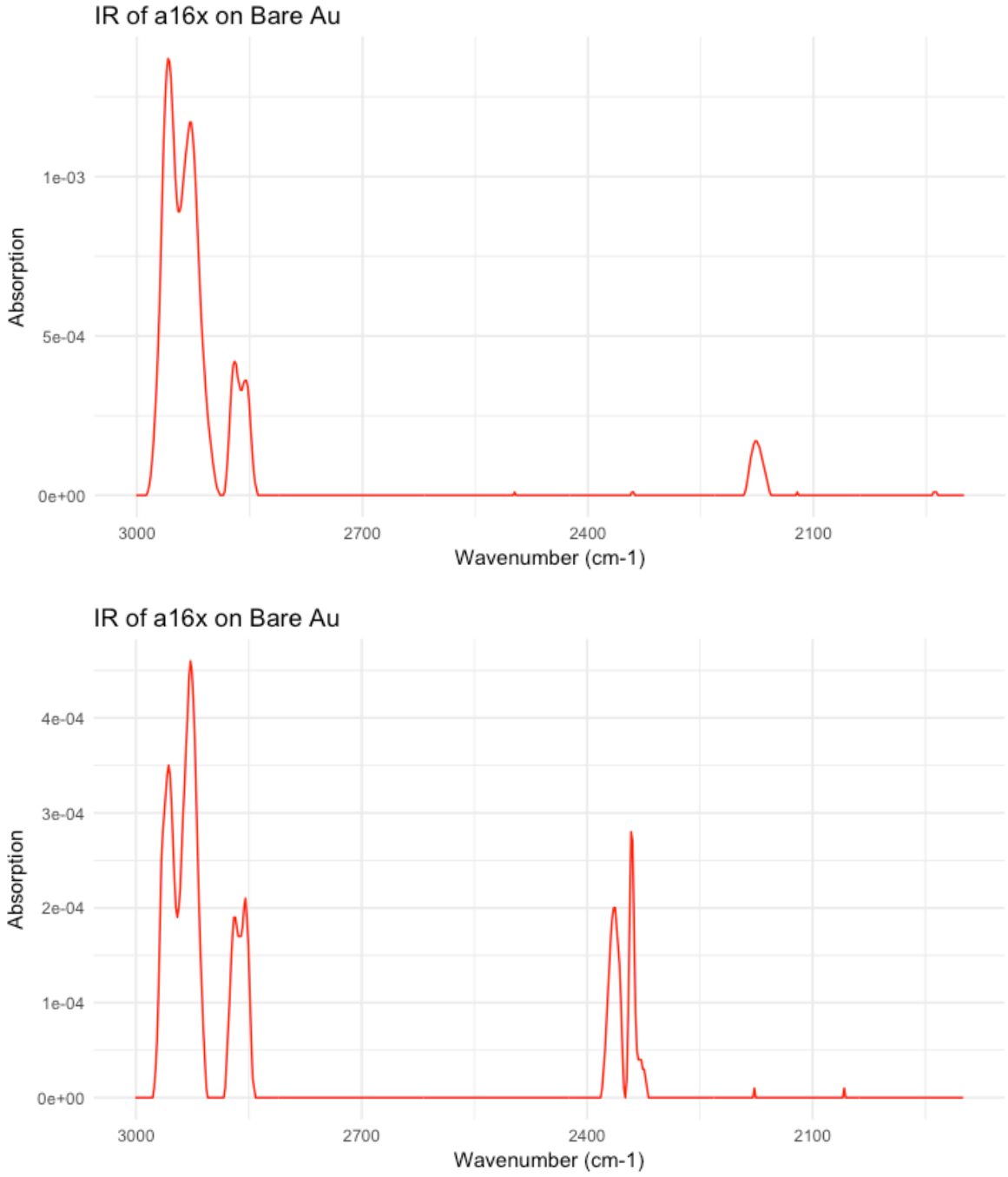
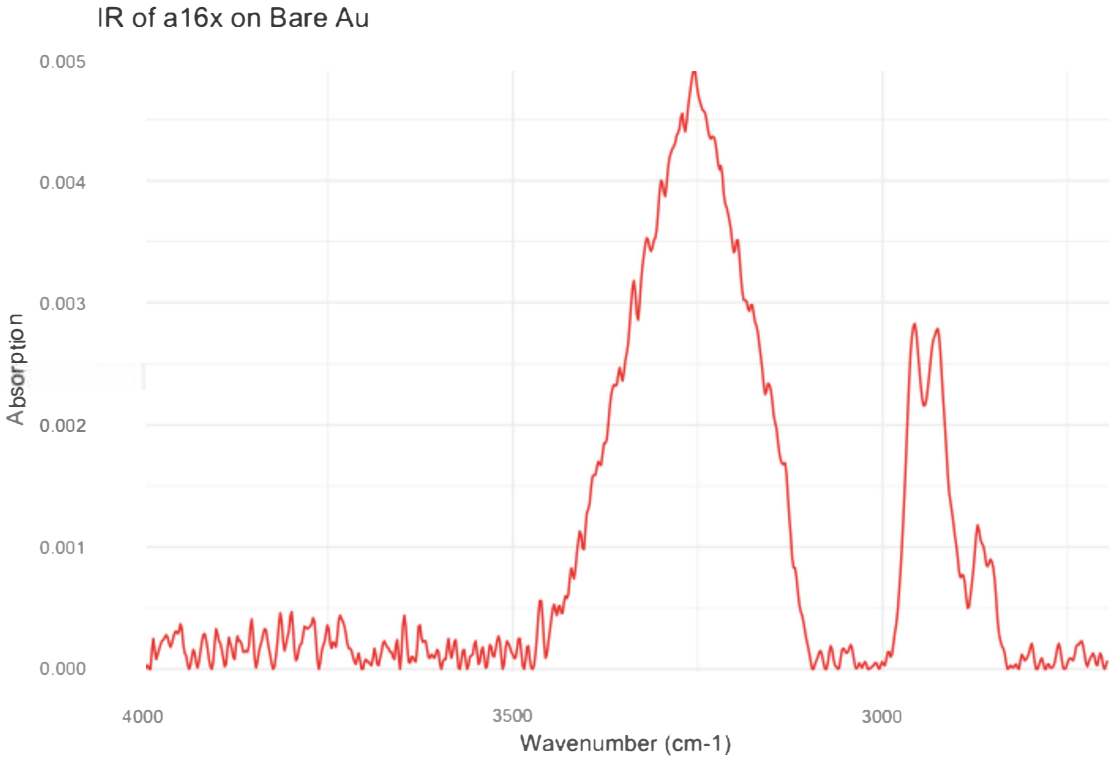


Figure 10: IR spectrum of a16x-functionalized gold surfaces utilizing the InSb detector. Top: First surface tested. Bottom: Last surface tested.

The methylene stretch is visible on the IR spectrum in the area corresponding to 2850-3000 wavenumbers; the strong azide peak, at 2150 wavenumbers. In the bottom spectrum a carbon dioxide stretch is visible from 2350-2400 wavenumbers on the last surface tested. This is a result of the IR chamber being improperly sealed. The in-house manual suction tool mount used to move the surfaces onto the GRAS-IR aperture does not properly seal the chamber and breaks the purge, allowing atmospheric carbon dioxide to enter into the chamber. These spectra contain two important observations: first, the extent of completion of the click reaction is variable across the prepared surfaces; second, in spite of this, peptide was able to be functionalized onto the surface.



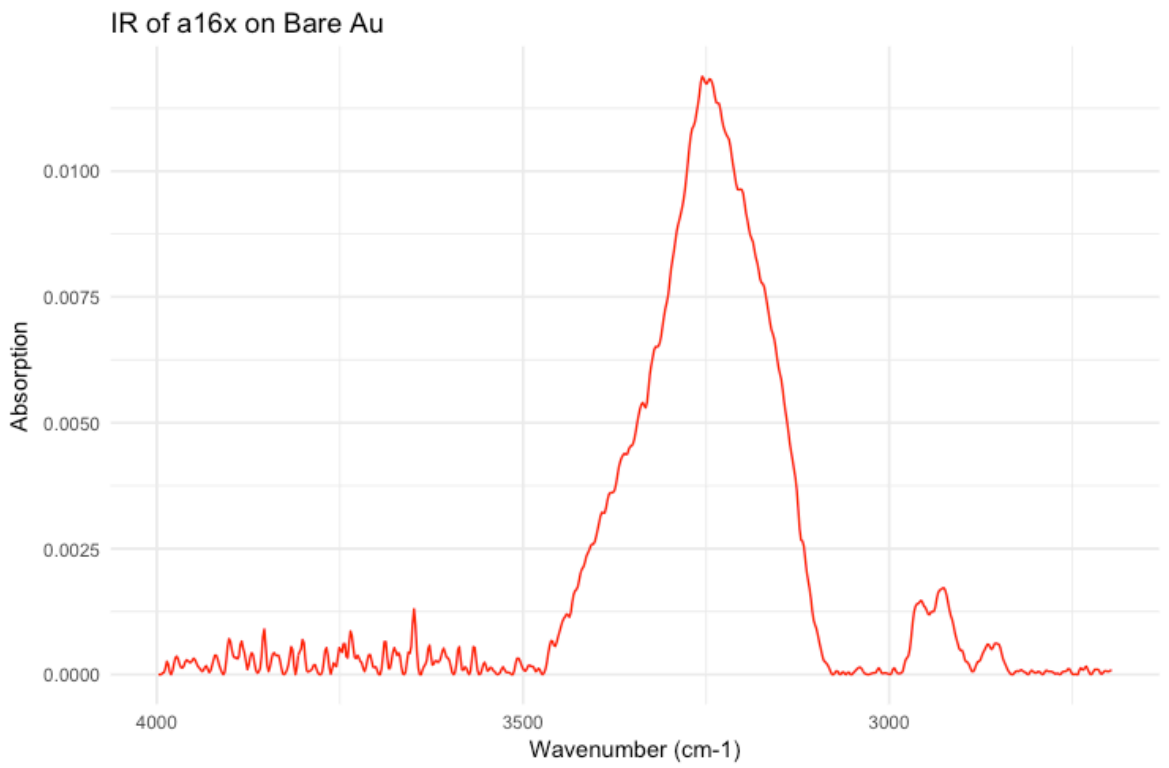


Figure 11: IR spectrum of a16x-functionalized gold surfaces utilizing the MCT detector. Top: First surface tested. Bottom: Last surface tested.

In the IR spectra collected using the MCT detector the broad secondary amine stretch is visible centered around 3300 wavenumbers. The first and third vibrational modes of water absorb near this range as well (at about 3400 wavenumbers) and the accumulation of atmospheric water in the sample chamber is evident by the shoulder near 3400 wave numbers in the final spectrum taken using the MCT detector. That being said, the large peak near 3300 wavenumbers visible in these spectra in addition to the methylene stretches visible in these and the InSb detector spectra point to the successful functionalization of the a16x peptide onto the surface.

IV. Discussion

The goal of this project is to advance the development of hybridized bio-inorganic materials. In order to do so, this project aims to demonstrate an attempt at a tunable, rationally designed system centered around the exploitation of electrostatic interactions at biological interfaces. The strategy presented in this work is to use a peptide whose electrostatic profile has been designed to complement one surface of a protein and chemically tether it to an inorganic surface via a self-assembled monolayer.

The protein Azurin was chosen for functional and practical reasons: first, its function is to perform an electron-transfer reaction and as such does not require a radical change in the protein's structure to be carried out; second, the protein naturally dimerizes in solution at a an alpha-helical interface, making it a convenient binding candidate for a small alpha-helical peptide. The peptide itself, a16x, is a small alpha-helical peptide composed primarily of hydrophobic residues so that it might complement the hydrophobic patch on the alpha-helical portion of Azurin's structure in addition to two unnatural residues which are necessary for tethering the peptide onto the surface. The surfaces in question are composed of a silicone discs which have been thermally deposited with a layer of chromium and a superficial layer of gold. The surfaces were prepared in the clean room facility of the Center for Nano and Molecular Science at the University of Texas at Austin. A self-assembled monolayer was constructed following a spontaneous adsorption of a thiol head group onto the gold surface. This monolayer was composed of long, alkyl chains. One quarter of these alkyl chains were terminated with Bromine functional groups which were then chemically converted to azide functional groups in anticipation of carrying out Huisgen cycloaddition.

As has been shown, Azurin was successfully purified using an e-coli vector. The secondary structure of both Azurin and the a16x peptide was verified via CD spectroscopy in a variety of solvents. UVPD mass spectrometry was also employed in order to determine the structural integrity of Azurin in different concentrations of mixed alcohol solvents. Together, these experiments determined the ideal solvent for both the Azurin protein and the a16x peptide; namely, 2:1 ethanol to water.

Infrared spectroscopy experiments of the peptide-functionalized surfaces were carried out in order to determine the success and extent of the Huisgen cycloaddition "Click" reaction. While the collected spectra reveal that the reaction proceeded unevenly on different surfaces (suggesting that the reaction conditions for the Click reaction have not been perfected for this system), these spectra also suggest that the a16x peptide was successfully functionalized onto the prepared gold surfaces. Future work in this project ought to develop a new, better mechanism for manipulating the peptide-functionalized surfaces inside of the IR spectrometer sample chamber in order to better maintain purge conditions, reduce atmospheric noise in the collected IR spectra, and minimize scratching to the surface samples.

This work has advanced the research efforts of the Webb laboratory by replicating previous experiments demonstrating the functionalization of an alpha-helical peptide onto an inorganic surface without compromising its secondary structure utilizing a new peptide system. Furthermore, the a16x peptide investigated herein was rationally designed in order to complement a specific protein, Azurin. This is a novel development from previous research efforts in the Webb laboratory. As such, it was also necessary to employ an experimental logic which could investigate mutually agreeable reaction and solution

conditions for both the protein and peptide. Finally, the ongoing collaboration with the Brodbelt group represents the first attempt to demonstrate complexing and binding specificity via UVPD mass spectrometry in a system as large as Azurin-a16x.

ONGOING AND FUTURE WORK

In collaboration with the Brodbelt group, ongoing UVPD mass spectrometry experiments aim to determine whether or not Azurin and the a16x peptide are complexing in solution and, furthermore, where on the Azurin structure the peptide is able to bind. Ongoing CD experiments in the Webb Laboratory aim to demonstrate the retention of the a16x peptide's secondary structure following functionalization onto the gold surface. Thin, quartz slides have been coated with very thin layers of chromium and gold via thermal deposition to this end.

Following the verification of complexing behavior between Azurin and the tethered a16x peptide, it is necessary to study the retention of Azurin's electron-transferring functionality. In order to accomplish this, electrochemistry techniques utilized to monitor the transferring of electrons via a chemical substrate are an option currently under consideration.

Notes

1. Eq 1.1 is the Poisson equation for the electrostatic potential, Eq 1.2 is the Boltzmann equation for ion density at the surface, and Eq 1.3 is obtained after some manipulation of the first two equations in order to obtain a charge density which can be subsisted into equation 1.1 in order to obtain the value of the electrostatic potential at any point near the surface with coordinates in the three dimensional cartesian coordinate plane x, y and z. In these equations Ψ represents the electrostatic potential, ρ the local charge density, c_i and c_i^0 the ion concentration at the surface and the bulk ion concentration, respectively, k_b is Boltzmann's constant, T is the temperature of the system, W_i is the work required to move a charge closer to the surface from an infinite distance, z_i is the valence of an ion, F is Faraday's constant, and q_e is the elementary charge of an electron.

2. Image generated using the PyMol visualization software.

3. The full units for CD are mili-degrees cm^2 dmol or 'molar ellipticity'. This is a result of the fact that incident light is *circularly* polarized and that the absorption of light is calculated with respect to the molarity of the sample.

4. Figure taken from the Webb research group's web page: http://webb.cm.utexas.edu/research/research_surface.html

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